

FMRI gene mutations in patients with fragile X syndrome and obligate carriers: 30 years of experience in Chile

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Summary

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID) and co-morbid autism. It is caused by an amplification of the CGG repeat (>200), which is known as the full mutation, within the 5'UTR of the *FMRI* gene. Expansions between 55–200 CGG repeats are termed premutation and are associated with a greater risk for fragile X-associated tremor/ataxia syndrome and fragile X-associated premature ovarian insufficiency. Intermediate alleles, also called the grey zone, include approximately 45–54 repeats and are considered borderline. Individuals with less than 45 repeats have a normal *FMRI* gene. We report the occurrence of CGG expansions of the *FMRI* gene in Chile among patients with ID and families with a known history of FXS. Here, we present a retrospective review conducted on 2321 cases (2202 probands and 119 relatives) referred for FXS diagnosis and cascade screening at the Institute of Nutrition and Food Technology (INTA), University of Chile. Samples were analysed using traditional cytogenetic methods and/or PCR. Southern blot was used to confirm the diagnosis. Overall frequency of *FMRI* expansions observed among probands was 194 (8.8%), the average age of diagnosis was 8.8 ± 5.4 years. Of 119 family members studied, 72 (60%) were diagnosed with a CGG expansion. Our results indicated that the prevalence of CGG expansions of the *FMRI* gene among probands is relatively higher than other populations. The average age of diagnosis is also higher than reference values. PCR and Southern blot represent a reliable molecular technique in the diagnosis of FXS.

1. Introduction

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability (ID) and co-morbid autism. It is characterized by an abnormal amplification of CGG repeats (>200) in the 5'UTR of the *FMRI* gene, located on chromosome Xq27.3 (Verkerk *et al.*, 1991; Hagerman *et al.*, 2009).

However, loss of function mutations (large deletions and point mutations) have also been associated with FXS, although they are far less documented (De Boulle *et al.*, 1993; Coffee *et al.*, 2008; Luo *et al.*, 2014). FXS affects individuals of both sexes with a wide spectrum of neurobehavioural phenotypes. Of these, 30–60% of boys, depending on the study, have autism spectrum disorders (Harris *et al.*, 2008; Budimirovic & Kaufmann, 2011; Abbeduto *et al.*, 2014).

A variety of genetic conditions related to the *FMRI* gene occur as a result of unstable CGG repeats, which are responsible for fragile X-associated genetic disorders (FXD), including FXS, fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated

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primary ovarian insufficiency (FXPOI) (Hagerman & Hagerman, 2013). A combination of molecular techniques including PCR and Southern blot (SB) are required to accurately determine the number of repeats as well as the methylation status of the promoter associated with expanded alleles in FXD (Hagerman, 2008).

Clinically, FXS is associated with a wide spectrum of neurodevelopmental phenotypes with cognitive functioning ranging from mild learning difficulties to severe ID (Harris *et al.*, 2008; Hagerman, 2013). The most common cognitive and behavioural characteristics identified in fragile X individuals are deficits in language and mathematics, social phobia, attention deficit, aggressive behaviour and hyperactivity. Physical features include large and prominent ears, elongated face with a prominent chin, hypermobility of the joints of the fingers, smooth skin and flat feet (Pieretti *et al.*, 1991).

Full mutation (FM) alleles exceeding 200 CGG repeats are associated with hypermethylation in the promoter region causing an absence of mRNA expression and subsequent deficit of the Fragile X Mental Retardation Protein (FMRP), essential for normal neurodevelopment (Pieretti *et al.*, 1991). Prevalence of FM in the general population has been reported to be higher in males than females, affecting 1 in 4000 males and 1 in 8000 females (Hagerman *et al.*, 2009; Song *et al.*, 2003; Hunter *et al.*, 2014).

Premutation (PM) is a medium size expansion (55–200 CGG repeats) mostly identified in unaffected carrier parents. Studies indicate a prevalence of 1 in 209 and 1 in 430 for females and males, respectively (Song *et al.*, 2003; Tassone *et al.*, 2012). In contrast to FM, PM alleles remain unmethylated and *FMRI* mRNA increases by up to five times the normal level, leading to mild to moderate FMRP production (Tassone *et al.*, 2000; Leehey *et al.*, 2003). Presence of high levels of mRNA produces a toxic effect on brain cells linked to late-onset disorders including FXTAS (Tassone *et al.*, 2004) usually affecting males >50 years of age (Rousseau *et al.*, 2011; Santa María *et al.*, 2014). Females carrying a PM are at an increased risk (~20%) of developing FXPOI, which usually occurs before 40 years of age (Schwartz *et al.*, 1994; Sherman *et al.*, 2000). Moreover, the risk of expanding from PM to FM in subsequent generations is determined by the CGG number, alleles >90 repeats have a 100% risk of expansion to a FM (Fisch *et al.*, 1995; Nolin *et al.*, 2003). On the other hand, intermediate or grey zone (GZ) alleles between 45 to 54 repeats overlap between PM and normal (<45) CGG size ranges, with a frequency of 1 in 112 males and 1 in 66 females (Tassone *et al.*, 2012). Expansions of the GZ are not clearly associated with a particular phenotype, but studies have suggested that carriers of these alleles have slightly

increased risk of the *FMRI*-related disorders FXTAS and FXPOI compared with normal alleles (Bennett *et al.*, 2010).

It is not uncommon that individuals with FXS may carry different CGG repeat allele sizes: some cells carry FM alleles while others carry PM alleles (Tassone *et al.*, 1999; Lokanga *et al.*, 2013). This instability of the CGG repeats within the *FMRI* gene is termed ‘size’ mosaicism and has been reported in 41% of males affected with FXS (Nolin *et al.*, 1994). In addition, some individuals exhibit ‘methylation’ mosaicism, in which some cells have fully methylated FM alleles while other cells possess unmethylated FM alleles (Merenstein *et al.*, 1996; Loesch *et al.*, 2012; Pretto *et al.*, 2013).

Previously, cases have shown that although mosaic individuals can have milder cognitive involvement, they can be at risk for developing FXTAS if their *FMRI* mRNA levels are elevated (de Vries *et al.*, 1996; Lokanga *et al.*, 2013). In post pubescent males methylation and size mosaicism has also been associated with higher IQ scores and lower phenotypic presentation, when compared to males with fully methylated FM (Tassone *et al.*, 1999; Lokanga *et al.*, 2013; Pretto *et al.*, 2014 a). Recently it has been shown that, *FMRI* mRNA and FMRP expression are directly correlated with percentage methylation of the *FMRI* allele. In addition, IQ scores were inversely correlated with percentage methylation and positively correlated with FMRP expression (Pretto *et al.*, 2014 b).

Chile has 16 million inhabitants, composed predominantly of a mixture of Amerindian and Spanish populations (Cruz-Coke, 1976). As in many other countries, the real number of individuals with CGG expansions remains unknown (Tejada *et al.*, 2014). The first case of FXS was reported in 1983 (Lacassie *et al.*, 1983). Since then, two small studies have reported 2 and 3% of FXS among individuals attending special schools (Aspillaga *et al.*, 1998; Alliende *et al.*, 2008). Although there are several large-scale studies reporting prevalence of FXS in different populations (Rousseau *et al.*, 2011; Tassone *et al.*, 2012), few analyses have been published in Latin America, underscoring the importance of our report. Our aim was to present 30 years of laboratory experience diagnosing FXS and its related disorders among individuals with ID referred for fragile X testing as well as in their family members.

2. Materials and methods

(i) Sample

A retrospective review was performed on 2321 cases referred for *FMRI* molecular testing conducted at the Cytogenetics and Molecular Laboratory at the

Institute of Nutrition and Food Technology (INTA), University of Chile from 1983 to 2013.

(ii) Methods

FMRI CGG-repeat size alleles were defined as normal size when between 5–44 repeats; intermediate or GZ when between 45–54 repeats; premutation when between 55–200 repeats and FM when >200 repeats. Fragile X Molecular Testing Guidelines of the American College of Medical Genetics, Standards and Guidelines for Clinical Genetics Laboratories (Monaghan *et al.*, 2013) were used.

(a) Cytogenetics

Initially (in 1983), fragile X diagnosis was based only on clinical and cytogenetic analyses (Lacassie *et al.*, 1983). However, by 1997 the Southern blot was introduced and positive cases were subsequently confirmed.

(b) PCR

Initially PCR was performed as described by Haddad *et al.* (1996) and applied only to males. PCR primers that flanked the trinucleotide repeats were used, with a product of 557 to 635 bp for normal size alleles visualized on silver-stained 6% polyacrylamide gel. Conditions were established such that FM that failed to amplify was then referred for SB analysis. PM alleles were not detected by this method. Since 2009, a new PCR assay was standardized, improving and refining the diagnostic testing by accurate amplification of fragments within the normal and PM range in both males and females (Saluto *et al.*, 2005). PCR was performed with minor modifications to the protocol described by Saluto *et al.* (2005). Briefly, PCR was based on 200 ng of gDNA to amplify the 5'UTR in the *FMRI* gene using fluorescent started primers c (GCTCAGCTCCGTTTCGGTTTCACTTCCGGT) and f (TGGAGGAGCTGGTGGTGGAAAGTGCGGGCT). Expand Long Taq polymerase (Roche) and Betaine (Sigma) were used to provide successful amplification of longer PM alleles of up to 130 CGG repeats in males and females. The amplified fragments were analysed in agarose gel at 2%. Normal males obtained PCR products between ~300–350 bp. In GZ and PM males, PCR products extended beyond 350 bp. Nevertheless, PCR cannot amplify long fragments within the FM range (the target region). Therefore the absence of amplification product in males indicated that a FM allele would be present. In heterozygous females, two sizes of PCR bands were obtained. However, if samples gave a single PCR fragment in females or failed to amplify alleles in males, SB analysis was used to measure FM expansion and differentiate between females who were

homozygous for a normal sized allele from females with a PM or FM heterozygous repeat expansion.

(c) CGG size amplification

Fragments greater than 350 bp long visualized by agarose gel (GZ and PM) were then sized using automated capillary electrophoresis ABI 3170xl (Applied Biosystems). Reactions were mixed with ROX 1000 size ladder and Hi-Di Formamide before being transferred to the Sequences Scan Analyzer. The fragment sizes were analysed using Gene Mapper 4.0.

(d) Southern blot

Direct analysis of the *FMRI* gene was performed using methods previously described (Alliende *et al.*, 2008). A double digestion of 20 µg of gDNA was performed using restriction endonucleases *EcoRI* and *EagI* overnight. After digestion and agarose gel electrophoresis, DNA was transferred to a nylon membrane Hybond N+ (Amersham). The membrane was hybridized with 12.3 StB probe provided by J. L. Mandel (INSERM, France). Finally the membrane was marked with chemiluminescence (PCR DIG Probe Synthesis Kit, Roche) and visualized by autoradiography (Fu *et al.*, 1991; Rousseau *et al.*, 1994; Alliende *et al.*, 1998).

(iii) Statistical analysis

Frequencies and percentages were used to describe overall findings. Logistic regression was used to predict the risk of having an affected child with FM. For that purpose, we firstly considered if the mother had a child with FM or not as a binomial outcome. In other words, if the mother had an affected child, then that was considered as '1', if not, then we computed it as '0'. Subsequently, we took into account the maternal total CGG length as a continuous and independent variable to carry out the logistic regression using the IBM SPSS software version 22; a *p*-value <0.05 was considered as significant. Finally, we used the probability given for the CGG length to delineate that relationship.

3. Results

A total of 2321 samples were referred to our laboratory for FXS testing over a 30-year period. Of these, 2202 (94%) were unrelated individuals with ID and clinical features of FXS whose ages ranged from 2 months to 25 years; 119 (6%) were family members of affected probands referred for cascade screening. The majority of patients were referred from a variety of medical specialists from other institutions in the country. The remaining patients were clinically

Table 1. *Distribution of FMR1 mutations with >55 CGG repeats detected in 194 FXS probands.*

Probands FXS	Males	Females	Total
Full mutation/fully methylated	135	30	165
Full mutation/partially methylated	1	0	1
Mosaics: full mutation/premutation	12	3	15
Premutation	5	1	6
Total	153	34	187

examined by paediatricians, clinical geneticists and neurologists from the Centre for Diagnosis and Treatment of Fragile X Syndrome (CDTSXF) at INTA.

(i) *FMR1 results among cases referred for fragile X testing*

Among 2202 probands with ID and clinical features of FXS, 194 (8.8%) presented a *FMR1* gene mutation, either a FM (n = 181), PM (n = 6) or GZ (n = 7). Table 1 shows the overall distribution of the different *FMR1* mutations with >55 CGG repeats according to gender. The most frequent mutation was a FM/fully methylated allele identified in 165 (7.5%) of subjects, of them 135 (81.8%) were males and 30 (18.2%) were females. Whether all patients with any type of FM are considered, the overall prevalence of *FMR1* mutations with >200 CGG repeats in the Chilean ID population is 8.2%.

Considering family history, 86 (44%) had a family history of ID and 108 (56%) had no previous history of ID in the family. The 194 FXS patients corresponded to 152 different families and 42 corresponded to families where there were two or more ID affected children studied at the same time. The largest family registered comprised 22 individuals with a *FMR1* expansion.

The average age at diagnosis for probands was 8.8 ± 5.4 years (mean calculated with the 166 cases in which the age at diagnosis was known). Table 2 shows that 77.7% of probands (129/166) were more than 5 years of age at diagnosis and only 22% (37/166) were diagnosed at <4 years of age.

(ii) *FMR1 results among cases referred for carrier detection*

Among the 119 relatives of FXS patients referred for carrier detection or cascade screening, 72 (60%) were diagnosed with an expansion in the *FMR1* gene; 61 were PM (54 females, seven males) and seven were FM females. Interestingly, two female obligate carriers presented different expansions in the *FMR1*

gene on both X chromosomes (compound heterozygous), they were daughters of non-consanguineous marriage, in which both parents were carriers of a PM. Additionally, an unmethylated FM male detected was part of a family study recently reported (Santa María *et al.*, 2014). Table 3 shows the number of offspring with a FM and PM with respect to the range of maternal total CGG repeats and the predicted risk. As can be seen, the percentage of children with a FM is greater for those mothers with a longer expansion of the CGG repeats. Indeed, all females with >100 CGG repeats had only children with a FM. The logistic regression confidently predicted this situation ($p = 0.011$), which means that even if the mother transmitted the PM allele, the risk of having a child with FXS is directly related to the number of CGG repeats in that allele.

4. Discussion

The present study is the first to estimate the prevalence of *FMR1* expansions in the Chilean population. Although, a number of studies have been carried out reporting prevalence of FXS in different populations (Crawford *et al.*, 2001; Puusepp *et al.*, 2008; Essop & Krause, 2013; Tejada *et al.*, 2014), knowledge in Latin America is relatively scant (Lacassie *et al.*, 1983; Florencia *et al.*, 2006; Christofolini *et al.*, 2009).

Overall, the diagnostic yield of *FMR1* variant expansions (FM, PM, GZ, size and methylation mosaics) was 8.8% (194/2202) in unrelated individuals with ID, and specifically, 8.2% (181/2202) had an allele with >200 CGG repeats. Of them, 6.7% were males and 1.5% females. The overall frequency detected is relatively higher compared to other ID populations, which show overall ranges in males between 1 and 5.2% (Crawford *et al.*, 2001; Essop & Krause, 2013). This paper reports a high frequency of *FMR1* mutations among Chilean ID patients, and it demonstrates the need for further testing in Latin American countries such as Chile. We cannot exclude a bias in the detection of the most affected patients and families with more than one member affected; due to economic and accessibility difficulties, only patients with a high suspicion of FXS are referred for molecular analysis.

Concerning mosaicism, the proportion of size mosaic detected having a PM and FM was low (15/194, 7.7% of FXS; Table 1) compared to 41% reported by Nolin *et al.* (1994) and other studies (Pretto *et al.*, 2014 a). This could be explained by methodological differences. Standard protocols used in this study may not be sufficiently sensitive to detect expansions of more than 130 CGG repeats or methylation mosaics (McConkie-Rosell *et al.*, 1993; Genc *et al.*, 2000). This result could also reflect a bias in the

Table 2. Distribution of FXS patients by age of diagnosis.

FXS patients	Age range (years)						Age unknown	Total
	0–4	5–9	10–14	15–19	20–24	25–29		
Males	33	52	29	20	2	3	24	163
Females	4	9	7	2	3	2	4	31
Total	37	61	36	22	5	5	28	194

Table 3. Transmission of FMR1 premutation repeats from 54 females.

Repeat size of maternal allele	Mothers (n)	Offspring with (n)		Offspring with an expanded allele (%)	Predicted risk of expansion to FM (%)
		PM	FM		
55–59	2	2	0	0	0.1–0.24
60–69	2	2	0	0	0.3–2.2
70–89	6	3	3	50	2.8–67.1
90–99	14	2	14	87.5	71.8–95.1
100–109	11	0	14	100	96–99.5
110–139	11	0	16	100	99.6–100
140–199	8	0	7	100	100–100
Total	54	9	54	87.5	

sample; mosaics that can produce some FMRP, depending on the methylation and the CGG allele size, are from less developmentally delayed patients and for economical and geographic reasons explained above, this population could be under-represented in this study (McConkie-Rosell *et al.*, 1993; Nolin *et al.*, 1994; Genc *et al.*, 2000; Tassone *et al.*, 2000; Loesch *et al.*, 2012; Pretto *et al.*, 2014 a; Santa Maria *et al.*, 2014).

As expected, a PM allele was detected in only 0.3% (6/2202) of individuals with ID (Table 1) supporting the notion that FM alleles are the most common cause of inherited ID. Moreover, as DNA testing was performed in blood and as these six PM patients showed signs of ID and behavioural problems, we cannot exclude that these patients correspond to a size mosaic pattern in other tissues (Pretto *et al.*, 2014 a).

The average age of diagnosis among probands was 8.8 ± 5.4 years, this estimation is considerably higher than the age reported in the USA, where it is around 3 years of age (Bailey *et al.*, 2009). One study in the US reported that 1 in 3 to 1 in 4 African–American children attending special education schools were diagnosed after 10 years of age. One possible explanation given was that social and cultural differences might be at play (Crawford *et al.*, 2002). In the context of Chile, the delay in diagnosis might more likely be related to lack of opportunity for diagnosis because of difficulties for families and health care programs in accessing genetic testing in Chile.

Our findings demonstrate a considerable number of unrelated FXS families, including a non-consanguineous family with two female sibling compound heterozygous patients with expansions on both alleles, inherited from unrelated PM parents (data not shown). This condition has been very rarely reported, only in five other cases. This information could be an indicator that the prevalence of this condition is similar to other populations and as a result, further studies are needed to confirm the prevalence of PM in the whole Chilean population.

Cascade screening and carrier detection in affected families allowed for the detection of 72 individuals with PM, FM or intermediate allele, including mothers of affected children, siblings, grandparents and second or third degree relatives. Because the *FMR1* mutation is rarely *de novo*, cascade screening from a proband is helpful to detect other cases in the family. In our study, the number of relatives tested by cascade screening was notably low indicating that many family members are not being tested or receiving appropriate genetic counselling. As a consequence, many young women will face a new pregnancy without the knowledge of a PM condition and will not be aware of the risk of developing an adult-onset disorder.

In this study PM alleles determined in 54 mothers were positively correlated with the risk of having FM offspring, confirming previous reports that an increasing likelihood for unstable transmissions in one generation with increasing repeat size (Table 3). It has been described that 55 CGG alleles can expand

to FM in a single generation (Biancalana *et al.*, 2004). Albeit two PM females with alleles in the 55–59 CGG range and two mothers with 60–69 CGG alleles did not have children with FM, this finding could be related to a sample size bias and the non-studied presence of AGG interruptions, which decrease the possibility of transmitting an expanded allele (Yrigollen *et al.*, 2012). Hence, it would be important to determine the number of AGG interruptions and the length of uninterrupted CGG repeats at the 3' end for better allele characterization and risk prediction.

The results presented in this study provide molecular information from a Latin American country about the complexity of a disorder with many molecular facets, including CGG repeat size and methylation, which can lead to the broad spectrum of clinical involvement in FXS. We emphasize that a detailed molecular diagnosis, particularly in those cases with mosaicism, could be used clinically to provide additional information for genetic counselling and expectations for the family. It could also guide clinicians and public health policy, specifically concerning early diagnosis.

5. Conclusion

This study evaluated the present status of fragile X diagnosis in Chile. Results indicate a need for education of health professionals from different disciplines regarding the importance of early detection, which could lead to minimizing or preventing the risk of transmission. Collaboration among professionals involved in the diagnosis of FXS in Chile has not been sufficient to break the chain of transmission.

There is a high probability of finding others affected or at risk of presenting *FMR1*-related disorders in a family when a *FMR1* expansion is detected. Support is improved with a confirmed diagnosis and may allow for the detection of more carriers in a family. This issue is essential for therapeutic approaches in FXS children, both in medical and cognitive outcomes, especially in families with more than one affected child.

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Declaration of interest

None.

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